

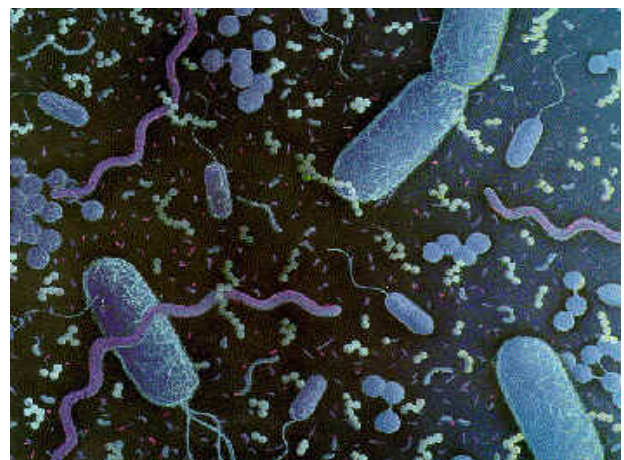
# Bacterial Flora of Osteoradionecrosis Detected by Molecular Techniques

Kjetil Pedersen

2006



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I would especially like to thank Lars Reime, who has been my companion in this work, for hours of brilliant teamwork, learning and fun.

I also acknowledge Emenike Eribe, his troubleshooting and advice has been most helpful.

## **Abstract**

The following is a report of a study undertaken to identify the bacterial species in bone samples from patients suffering from osteoradionecrosis. This was done using polymerase chain reaction (PCR), cloning and sequencing techniques, where 16S rRNA was the gene used for analysis. The results from two patient samples will be presented. As far as we know, this is the first study that includes molecular genetic techniques to detect bacteria associated with osteoradionecrosis.

## **Introduction**

Patients who have been diagnosed with cancer in the head or neck region and subsequently been treated with radiation therapy, may experience a number of complications or side effects. These complications include mucositis, dysphagia, alterations of taste, infections, dermatitis, xerostomia, increased risk of caries, trismus and osteoradionecrosis (ORN). Some of these problems are temporary, whereas the risk of ORN is lifelong and may occur many years after irradiation (1).

Some discussion on previous findings will be included, but this project has mainly involved many hours of laboratory work.

The textbook definition on ORN has been: “An area >1cm of exposed bone in a radiated area, showing no signs of healing within 6 (3-6) months.” However sometimes one can see ORN and intact mucosa intraorally, so a newer definition has been suggested by Støre and Boysen (2): “Radiological evidence of bone necrosis within the radiation field, where tumor recurrence has been excluded.”

Chief factor responsible for ORN is the amount of radiation directed through bone, but poor nutrition, oral health and large alcohol consumption seems to contribute to development of this serious condition.

The most accepted concept of ORN etiology, was formulated by Marx (3). ORN is caused by intraosseal ischaemia, multiple embolization, extensive tissue hypoxia and secondary cell destruction.

The traditional viewpoint is that presence of bacteria in ORN-bone samples represents secondary infection or superficial contamination. Some suggest however that ORN may have a contributing infectious etiology (3).

It seems that the radiation has deleterious effects on osteocytes, osteoblasts and endothelial cells, causing reduced capacity of bone to recover from injury, which may come in the form of trauma, or infection by advancing periodontal disease, periapical inflammation (4) or through a haematogenic pathway (3). ORN has also been found to occur where no known injury can be identified (1).

It has been estimated that about 50% of the oral microflora is uncultivable (5), so it is reasonable to assume that a wide range of bacteria could be found in ORN samples. Our purpose was to try to capture all bacterial DNA by using PCR technique with universal primers for the 16S rRNA-gene. This 1500 base pair long gene found in all bacteria has proved to be a well suited gene for identifying and classifying bacteria (5). The purpose of this project was to analyze the bacterial microflora in bone samples from patients suffering from ORN of the jaw, using PCR (polymerase chain reaction), cloning and sequencing techniques. Since ORN has such serious consequences, so any new knowledge that could shed some light on its etiology will be of value.

In summary our aims were:

- To test molecular genetic techniques such as PCR, cloning and sequencing, for bacterial identification from bone samples.
- To identify predominant bacteria present in ORN and see if there are any especially pathogenic bacteria present.

## **Materials and methods**

First I would like to give a brief overview of the different steps in our work and explain their main purpose, before presenting a more detailed account of each technique with its specific protocol. Before any of the steps below were initiated, an application was written and sent to the appropriate ethical committee, Regional Etisk Komite Sør, which approved the study.

### **Brief overview**

1. Sample collection from patients suffering from ORN of the jaw.
2. Sample grinding and extraction of bacterial DNA. Here we wished to capture the bacterial DNA and wash out all other cell material and debris.
3. PCR with universal primers to detect bacterial DNA. To give us sufficient DNA material for further analysis.
4. Electrophoresis. After the extraction process we could not tell if we actually had any bacterial DNA in the tubes, so by performing gel electrophoresis we could verify that there was bacterial DNA present.
5. Cloning of the PCR product into electrocompetent cells. After the PCR- reaction each sample contains millions of equally long DNA-strands representing a number of different bacteria. By inserting these strands into cloning cells where each cell can absorb only one DNA-segment we are able to separate the different DNA-strands.
6. PCR with DNA from cloning cells. To give us the DNA- material needed for sequencing.
7. Gel electrophoresis. To verify that there is DNA present after the last PCR-reaction.

8. Sequencing. Here the DNA-strands are analyzed so that we are given the specific order of the bases in the DNA we started out with.
9. Data analysis. Finally we wish to compare “our” DNA- sequences with known bacteria available in databases for species identity and closest relatives.

### **1. Sample collecting:**

Tissue samples were collected by Dr. Geir Støre at Rikshospitalet. The samples were collected from patients who needed to remove segments of the jaw as part of the treatment of ORN. Resections were made from the body of the mandible and some specimens were obtained using sterile trepan burrs (3 mm) wide. The bone material was put in a Tris-HCL buffer solution in sterile containers and stored at -20°C until the analysis began.

### **2. Sample grinding DNA extraction:**

By grinding the bone samples to a fine powder, the involved bacteria were more available for analysis. This powder was collected in sterile tubes and made the starting point for capturing bacterial DNA. It proved quite difficult to extract the DNA and have a successful PCR- reaction, and we tried numerous protocols before we were able to find some that worked properly. By the help of different reagents we basically broke down the proteins and the cell walls and washed out all these components except for the DNA which was left in a tube and stored at 4°C before PCR.

It is important to prevent contamination of the samples, either from the oral cavity of the patient, the surgical operators or through the first step of grinding the samples. This second step was performed in a ventilated compartment and the results from samples that proved to contain compost bacteria or staphylococcus, were discarded.



*Protocol:*

Grind samples individually, using Sterile mortars and liquid nitrogen, dissolve powder in 0.1M tris HCl buffer and put in Eppendorf tubes. Perform this first step in a hood. Extract bacterial DNA using one of two different techniques.

1: Use reagents from MasterPure DNA purification Kit (Epicentre Technologies) as follows: 10 µl proteinase K added to 100 µl sample and incubated two hours at 55°C. Add 300 µl “tissue and cell lysis solution”, vortex, incubate 15 min. at 65°C and vortex every 5<sup>th</sup> min. Spin down (in sentrifuge), put tube on ice, add 150 µl “MPC Protein precipitation reagent” and vortex for 10 sec. Sentrifuge (15,000 rpm) at 4°C for 10 min, transfer supernatant to an empty tube, discard pellet and repeat the step. 500µl of isopropanol is added and the closed tube is turned upside down 30-40 times. Sentrifuge as before and gently pour off isopropanol without loosing the pellet, before washing the pellet twice with 96% and 70% ethanol. Remove ethanol and dry the pellet briefly at 37°C before dissolving the DNA pellet in 35 µl tris HCL.

2: Using ChargeSwitch Forensic DNA Purification Kit (Invitrogen) and follow the guidelines of the producer.

### **3. Amplification of 16S rRNA genes by Polymerase Chain Reaction, (PCR):**

PCR is a well established technique for amplifying selected DNA sequences (6).

The 16S rRNA genes were amplified under standardized conditions using a universal primer set (9F, forward primer-5'-GAG AGT TTG ATY MTG GCT CAG-3'; 1541R, reverse primer 5'-GAA GGA GGT GWT CCA RCC GCA-3') (7). Primers were synthesized commercially (Operon Technologies, Alameda, CA). The PCR primers do not necessarily cover all bacterial species. Nevertheless, a wide range of phylogenic types has been obtained in our study and previous studies by using this universal primer set.

## Bacterial flora detected in osteoradionecrosis

After the extraction process we had one tube for each of the samples containing the DNA of (hopefully) several different bacterial species (fig. 1). Not knowing what concentration of DNA we had in each of these tubes we used different amounts of this “DNA solution” in several PCR-reactions for each sample.

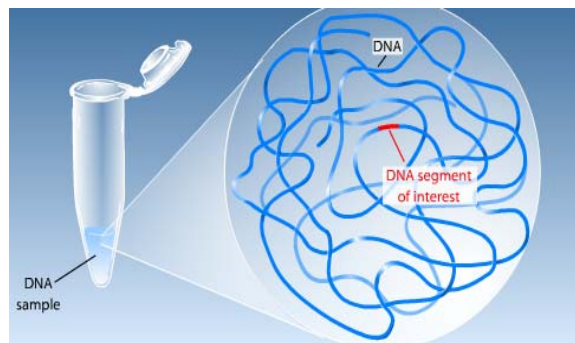


Figure 1. DNA from clinical samples.

When setting up the PCR-reaction we also had one positive and one negative control tube, to check that the reaction ran properly and that we didn't have any contamination.

The “PCR-mix” contained Taq polymerase, the enzyme which actually builds the DNA-strand, nucleotides or the DNA building blocks, the primers and buffer solution (fig. 2). This mixture was spread to different tubes before the DNA was applied.

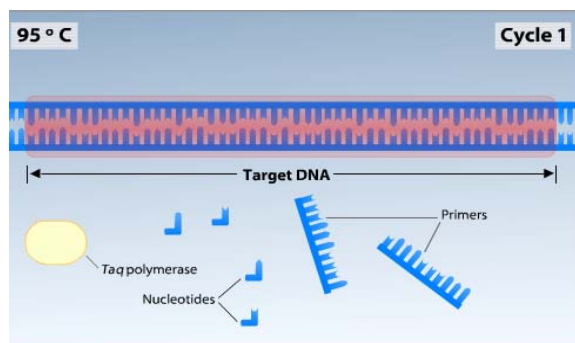


Figure 2

The First step in the three step cycle of PCR is to heat up the mixture so that the DNA is denatured (fig. 3), meaning the double stranded DNA becoming separated.

The separation of the double strand allows a small segment of DNA, called a primer, to attach. The temperature at which this happens is specific for each type of primer. Annealing of the primer is the second step in one cycle (fig. 4).

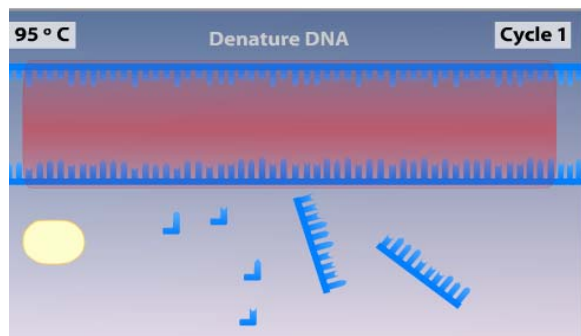


Figure 3

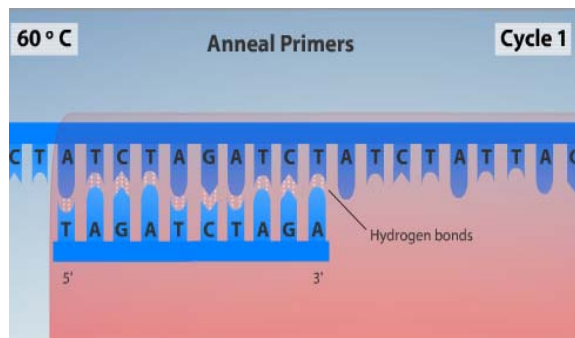


Figure 4

The final step of one cycle is to polymerize DNA by the help of heat resistant bacterial polymerase at 72°C (fig. 5). In this first cycle two copies of the DNA of interest are made, and in the second cycle another four copies are produced, and so on through many cycles. By repeating this process 30 times several million copies of DNA are produced (fig. 6). It is important to remember that in this first PCR- reaction all bacteria present in the original sample may not be represented.

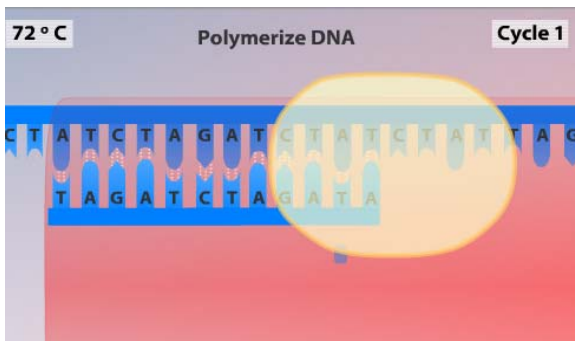


Figure 5



Figure 6



Figure 7. Thermocycler used for the PCR technique.



Figure 8. The PCR protocol.

*Protocol:*

PCR was performed in thin-walled tubes with a Gene amp PCR system 9700 (ABI, Foster, CA). 4, 2, 1 and 0.1 µl of the lysed sample were added to 4 different tubes containing the reaction mixture (final volume, 50 µl) containing 20 pmol of each primer, 40 nmol of deoxynucleoside triphosphates, and 1 U of HotStarTaq Master Mix (Qiagen). In a hot-start protocol, the samples were preheated at 95°C for 4 min, followed by amplification under the following conditions: denaturation at 95°C for 45 s, annealing at 60°C for 45 s, and elongation at 72° for 1.5 min, with an additional 15 s for each cycle. A total of 30 cycles were performed; this was followed by a final elongation step at 72°C for 15 min. The results of the PCR amplification were examined by electrophoresis in a 0.9% agarose gel.

**4. Electrophoresis:**

Using gel electrophoresis, we wanted to Check PCR products for the presence of DNA. By Applying DNA to a gel one can separate DNA of different lengths. Since DNA is negatively charged it will move towards the positive side of the electric source in the tray, and because of the resistance in the gel, shorter segments will move faster through the gel compared to larger segments.

*Protocol:*

Mix 5 µl PCR.product with 5 µl 10X gel loading buffer. Apply these 10 µl to the well of a 0.9 % agarose gel lying in an electrophoreses-tray filled with 1X TAE-buffer. Connect electricity, 200 V for about 20-25 min. Immerse gel in ethidium bromide solution for 30 min., rinse thoroughly in tap water and put gel onto a short wavelength UV-light board. DNA should show as clear bands in the gel. Take a picture of the gel or note down which of the columns that contains DNA.

## 5. Cloning of the PCR-product:

Before cloning, we performed a purifying step by taking all of the remaining PCR-product and performing an electrophoresis, where the band containing the DNA was cut out and then rinsed. This purified DNA was the DNA we used for cloning. DNA cloning is a method for isolating a particular sequence of DNA from a mixture of DNA sequences.

First we need to insert it into a vector, usually a modified phage or plasmid (fig.9 and 10).

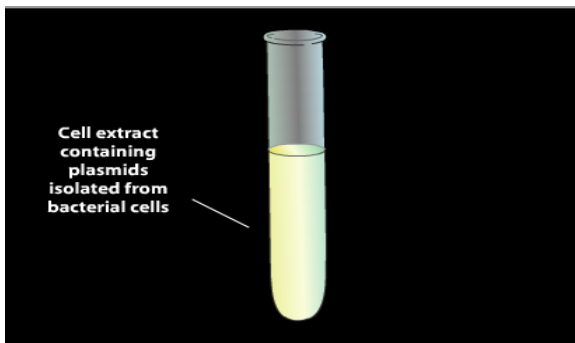


Figure 9

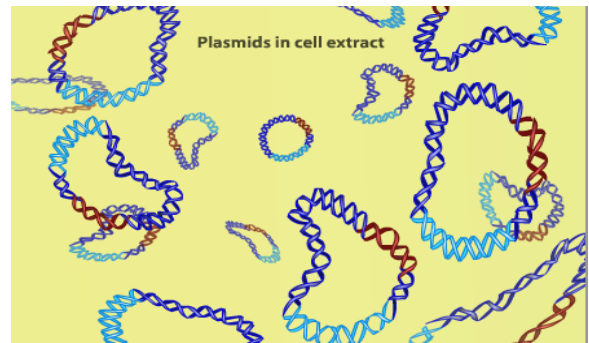


Figure 10

A vector usually contains three elements (fig. 11), a cloning site where the DNA fragment can be inserted, a drug-resistance gene, which destroys antibiotics, in this case kanamycin, to allow selective growth of the host cell, and a replication origin to allow the plasmid to replicate in the host cell. By using a restriction enzyme we can cleave the vector at the cloning site and then introduce foreign DNA which has been cut using the same enzyme (fig. 12).

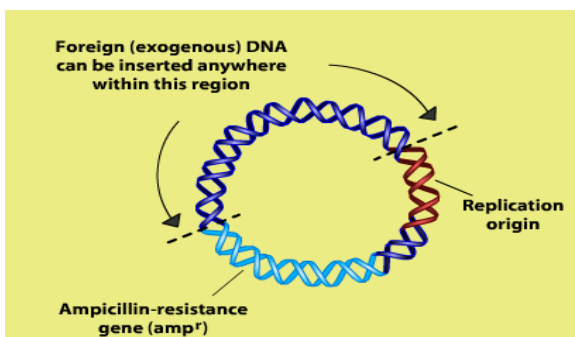


Figure 11

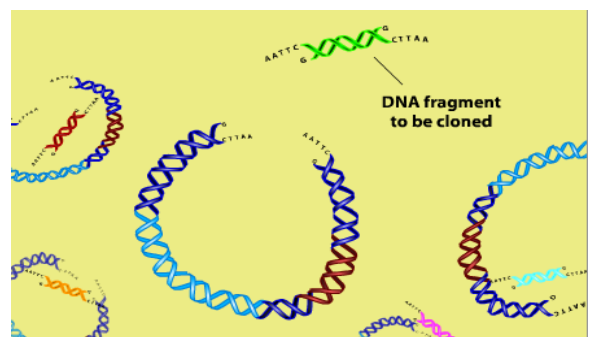


Figure 12

When foreign DNA is sealed into the plasmid we have created recombinant plasmids (fig. 13) where each plasmid now contains a unique fragment of DNA. Modified *Eschericia Coli* is added and a through a process called transformation a few of these take up a recombinant plasmid (fig. 14).

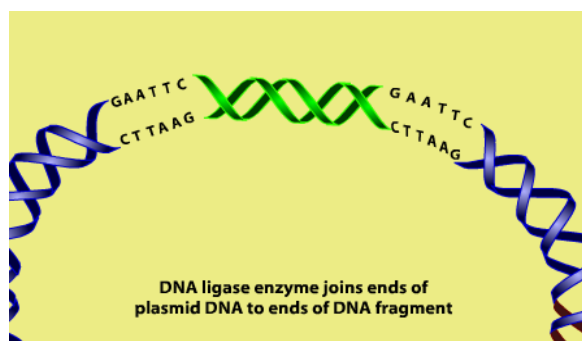


Figure 13

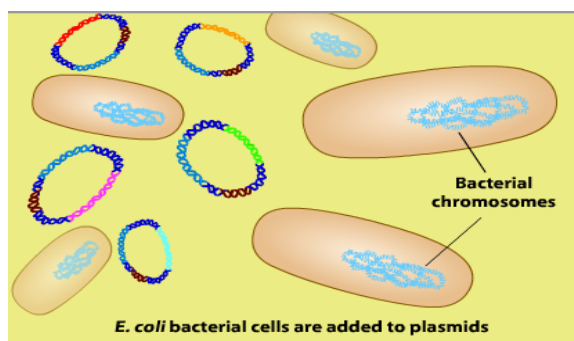


Figure 14

The bacterial cells are poured onto agar containing kanamycin and incubated at 37°C (fig. 15). Only the cells that contain the recombinant plasmid will be resistant to the antibiotic and thus allowed to grow (fig.16).

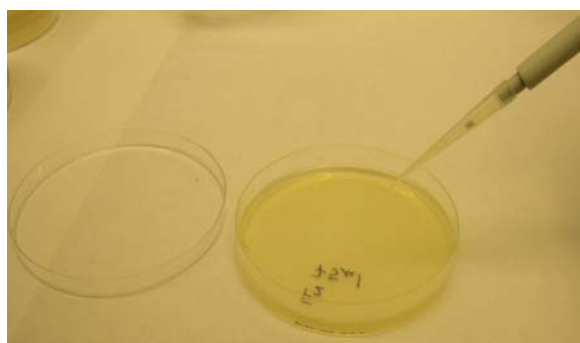


Figure 15. Growth of cloning cells.

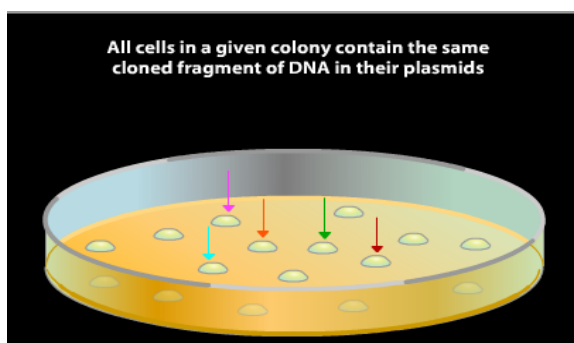


Figure 16

After incubation for 24 hours, individual colonies are picked (fig. 17) and streaked onto a new gridded agar tray (fig. 18), allowing us to count specific colonies. We used two new trays for each sample giving 96 colonies, incubated for 24 hours, picked the colonies and stored them in a buffer solution.

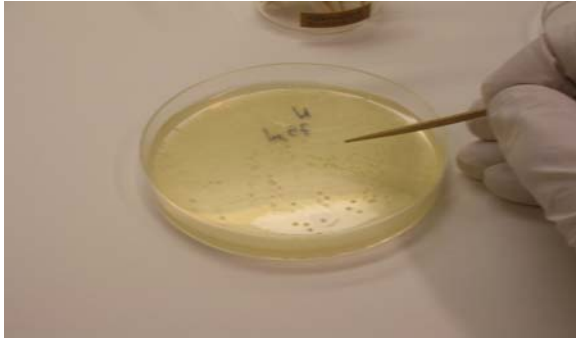


Figure 17

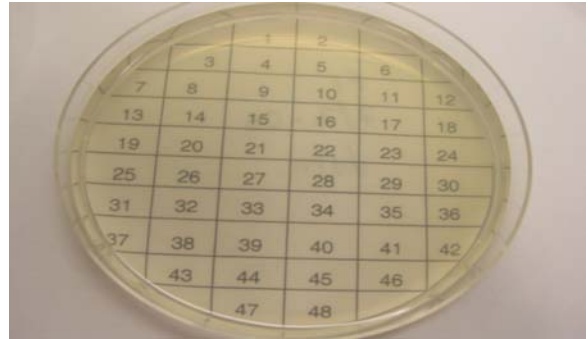


Figure 18

### *Protocol:*

Apply 20  $\mu$ l PCR product to Agarose gel with large wells, run electrophoresis and put gel in ethidium bromide for 30min. Cut out the part of the gel where band appears and use Qiagen Gel Extraction kit to purify before cloning. Cloning of PCR-amplified and purified DNA was performed with the TOPO TA cloning kit (Invitrogen) according to the instructions of the manufacturer. Transformation was done with competent *E. coli* TOP10 cells provided by the manufacturer. The transformed cells were then plated onto 4 Luria-Bertoni agar plates supplemented with kanamycin (50  $\mu$ g/ml) and Xgal, and the plates were incubated overnight at 37°C. Pick the clear colonies and streak them onto a gridded plate (96 colonies per sample), then incubate overnight. Each colony was placed into 40  $\mu$ l of 10 mM Tris. Correct sizes of the inserts were determined in a PCR with an M13 (-20) forward primer and an M13 reverse primer (Invitrogen). Prior to sequencing of the fragments, the PCR-amplified 16S rRNA fragments were purified and concentrated with Microcon 100 (Amicon, Bedford, MA), followed by use of the QIAquick PCR purification kit (Qiagen, Valencia, CA).



## **6. Clone collecting and PCR:**

After incubation, the E-coli colonies needed to be transferred to a solution to be available for further analysis. Individual colonies were dissolved in numbered tubes corresponding to colony number on plate. The type of vector will dictate the type of primer needed to be used in the PCR- reaction.

### *Protocol:*

Scrape the cells from the gridded plate and suspend in 40 µl Tris/HCL then perform a new PCR with each collected clone using M13F and M13R primers and 1 µl of solution containing the cloned cells.

## **7. Electrophoresis:**

Verify PCR products using gel electrophoresis. Here we used a premade Gel from Invitrogen containing 96 wells, to save some time, and the PCR- samples giving a positive result here were ready to be prepared for sequencing.

### *Protocol:*

Mix 5 µl of PCR-product, 5 µl of 10X gel loading buffer and 10 µl of H<sub>2</sub>O, and apply this to a well of the E-Gel. Connect the E- Gel to the electricity for 8 min. and put onto a UV-light table and take picture of this plate.



## 8. 16S rRNA gene sequencing:

Before sequencing, the samples are purified using a set of enzymes (exonuclease and phosphatase) which breaks down the other structures in the tube except for DNA. The samples are then run through a process which is similar to the PCR- technique in some respects. Copies of the DNA are made, but in the extension process some of the nucleotides are modified. On the first picture below, regular nucleotides are shown (fig. 19).

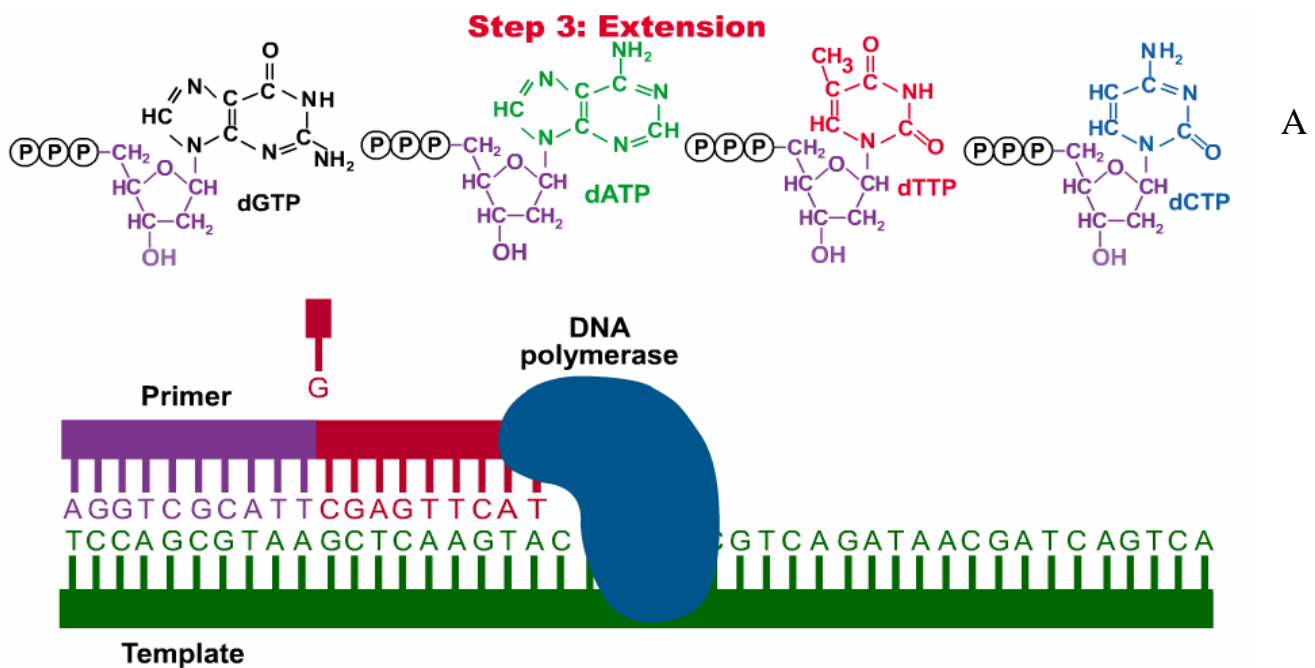


Figure 19

small percentage of the building blocks are labelled with a fluorescent dye, and are missing the 3' OH-group (fig. 20), which will terminate the extension step and result in many copies of the same DNA- segment with varying lengths.

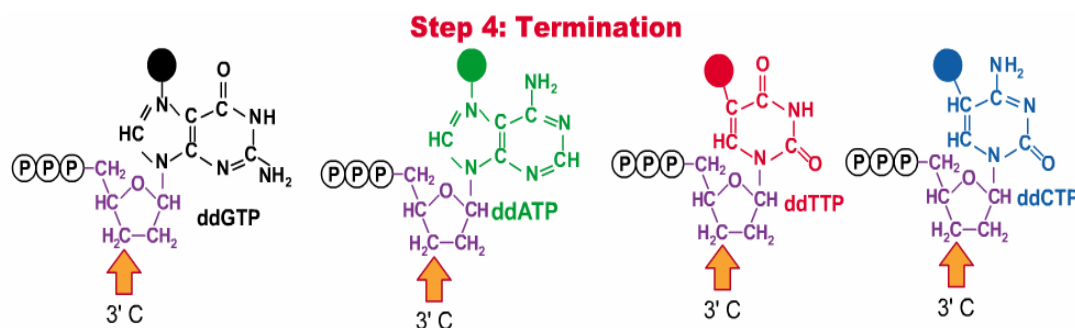


Figure 20

By chance we will have many copies of the same lengths in the solution where each sequence has a labelled nucleotide at the end (fig. 21), specific for that type of nucleotide. This solution is then filtered in a special way, dried completely, to make sure that everything but DNA is removed, before DNA is resolved and put into the sequencing machine. This machine is basically a high Voltage electrophoresis machine with capillaries filled with liquid polymer (fig. 23). Shorter sequences will move faster through the capillaries and this way sequences are sorted in order of increasing length.

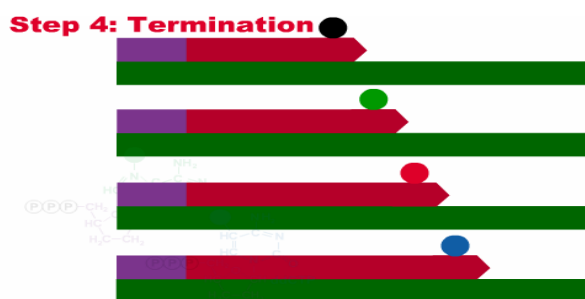


Figure 21



Figure 22



Figure 23

At the end of the capillaries a laser beam (fig. 22) illuminates the passing sequence which in turn emits light of a specific wavelength corresponding to a specific nucleotide (A, G, C or T) at the end of the sequence. This short glimpse of light is registered, recorded and the DNA- sequence is then available for analysis.

*Protocol:*

72 different (?) DNA strands, for each original sample were prepared for sequencing. Clean up PCR products using Exonuclease 1/Shrimp Alkaline Phosphatase (SAP) Dilution protocol. Purified PCR-amplified 16S rRNA inserts were sequenced using an ABI Prism cycle sequencing kit (BigDye Terminator Cycle Sequencing kit with AmpliTaq DNA Polymerase FS; Gene amp PCR system 9700 (ABI)). The primers used for sequencing have been described previously (Paster et al. 2001). Quarter dye chemistry was used with 80  $\mu$ M primers and 1.5  $\mu$ l of PCR product in a final volume of 20  $\mu$ l. Cycle sequencing was performed with a Gene amp PCR system 9700 (ABI) with 25 cycles of denaturation at 96°C for 10 s, annealing at 55° for 5 s, and extension at 60°C for 4 min. The sequencing reactions were run on an ABI 3100 DNA sequencer (ABI).

## **9. Data analysis of unrecognized inserts.**

The raw material from the sequencing process was processed by using a software called Sequencher. First we cut away DNA known to belong to the vector, then primers were “inserted” so that it was possible to orientate where among the bases our gene of interest started. The given order of the bases was then checked against the graphical layout from the sequencing machine (fig. 24). This graphical layout is a direct expression of the lights registered at the end of the capillaries.

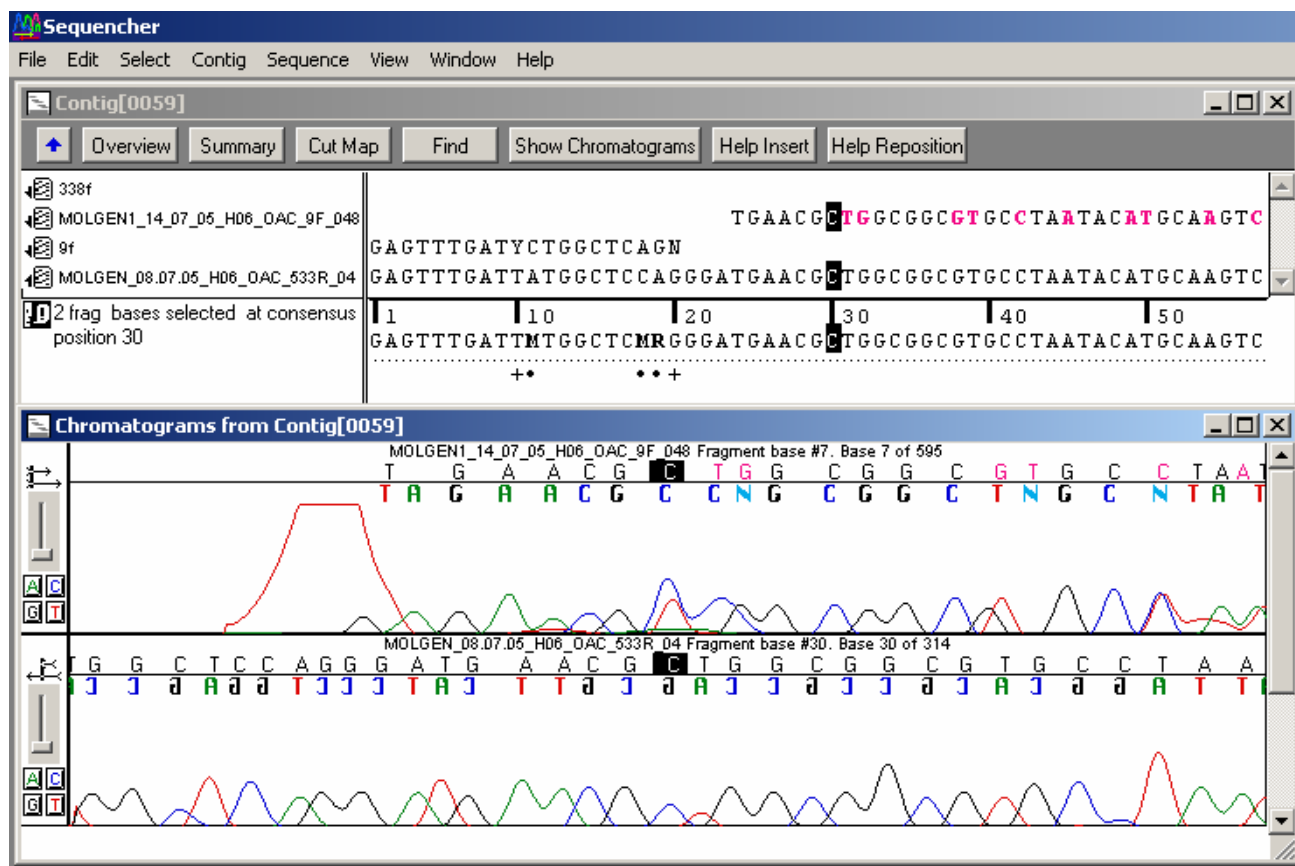


Figure 24

A sequence of approximately 500 bases was obtained first to determine identity or approximate phylogenetic position. Full sequences of about 1,500 bases were obtained by using five to six additional sequencing primers (5) for those species deemed novel. For identification of closest relatives, the sequences of the unrecognized inserts were compared to the 16S rRNA sequences of over 10,000 microorganisms in our database and over 100,000 sequences in the Ribosomal Database Project (8) and the GenBank databases. Our cutoff for species differentiation was 2%, or approximately 30 bases for a full sequence. The similarity matrices were corrected for multiple base changes at single positions by the method of Jukes and Cantor (9). Similarity matrices were constructed from the aligned sequences by using only those sequence positions for which data were available for 90% of the strains tested. Phylogenetic trees were constructed by the neighbor-joining method of Saitou and Nei (10). TREECON, a software package for the

Microsoft Windows environment, was used for the construction and drawing of evolutionary trees (11). We are aware of the potential creation of 16S rRNA chimera molecules assembled during the PCR (12). The percentage of chimeric inserts in 16S rRNA gene libraries ranged from 1 to 15%. Chimeric sequences were identified by using the Chimera Check program in RDP, by treeing analysis, or by base signature analysis. Species identification of chimeras was obtained, but the sequences were not examined for phylogenetic analysis.

### **Nucleotide sequence accession numbers.**

The complete 16S rRNA gene sequences of clones representing novel phylotypes defined in this study, sequences of known species not previously reported, and published sequences are available for electronic retrieval from the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers shown in Figs. X.

Due to some technical difficulties with the final step of sequencing only two out of eight samples were ready to be checked against known sequences at the time this report was made.

## **Results and discussion**

Patient 1: 56 year old male, cancer of the tonsilla on left side, received maximum radiation dose, developed ORN after extraction of 38, exposed bone.

Patient 2: 74 year old male, cancer of the tonsilla on left side, received maximum radiation dose, developed ORN with patologic fracture and exposed bone after extraction of 48.

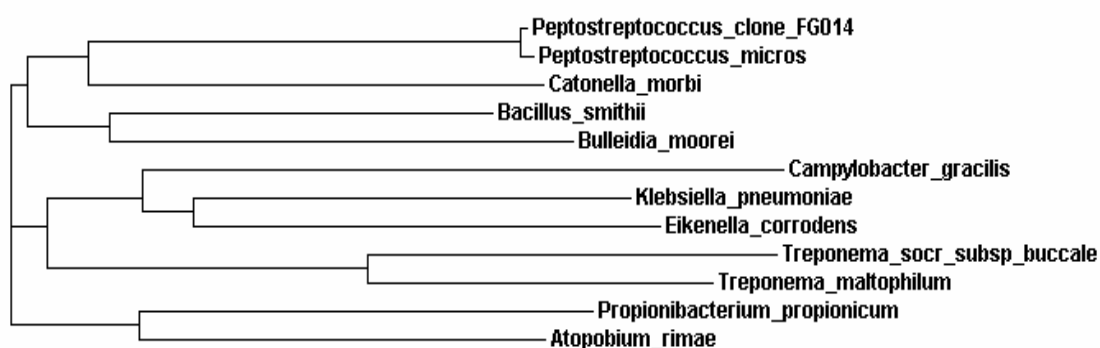


Figure 25. Phylogenetic tree representing bacteria detected from patient 1.

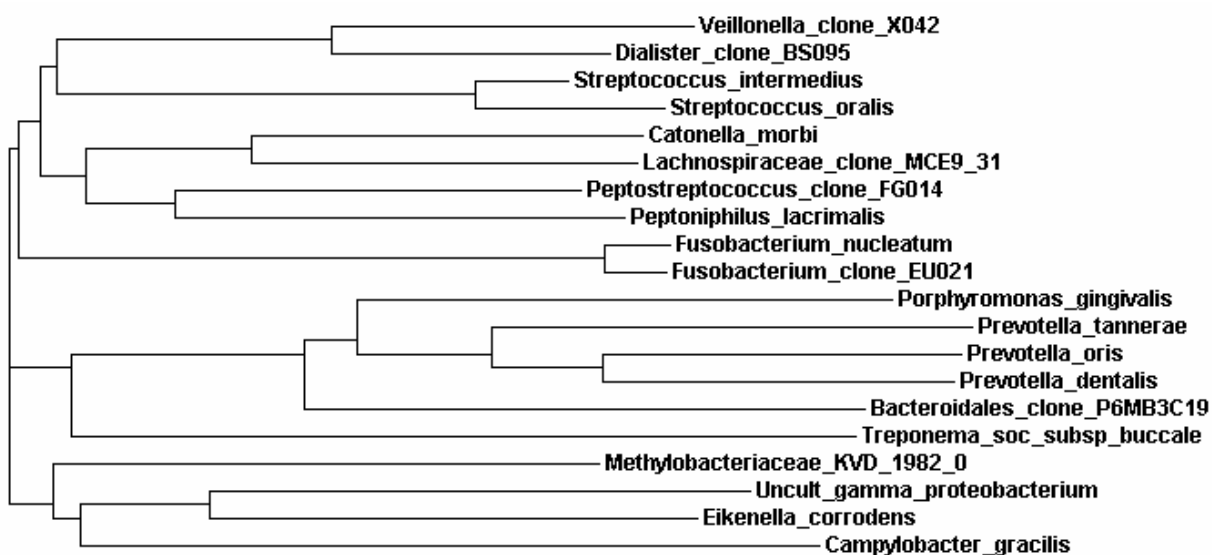


Figure 26. Phylogenetic tree representing bacteria detected from patient 2.

	Patient	
	1	2
Type of bacteria	Times represented in clones	
<i>Atopobium rimae</i>	4	
<i>Bacteroidales</i> genomsp. Clone P6 MB3C19		1
<i>Bacillus smithii</i>	1	
<i>Bulleidia moorei</i>	1	
<i>Catonella morbi</i>	9	3
<i>Campylobacter gracilis</i>	17	1
<i>Clostridia bacterium</i>		1
<i>Dialister</i> sp. oral clone BS095		2
<i>Eikenella corrodens</i>	2	1
<i>Fusobacterium</i> sp. oral clone EU021		7
<i>Fusobacterium nucleatum</i>		8
<i>Klebsiella pneumoniae</i>	1	
<i>Lachnospiraceae</i> oral clone MCE9-31		2
<i>Methylobacteriaceae</i>		2
<i>Peptinophilus lacrimalis</i>		1
<i>Peptostreptococcus</i> sp. oral clone FG014	9	7
<i>Peptostreptococcus micros</i>	13	
<i>Porphyromona gingivalis</i>		6
<i>Prevotella dentalis</i>		1
<i>Prevotella oris</i>		1
<i>Prevotella tannarae</i>		2
<i>Propionibacterium propionicum</i>	3	
<i>Streptococcus intermedius</i>		11
<i>Streptococcus oralis</i>		1
<i>Treponema socranskii</i> subsp. buccale	10	1
<i>Treponema maltophilum</i>	1	
<i>Veillonella</i> clone X042		1
<i>Uncultured gamma proteobacterium</i>		1
Total number of sequences	68	61
number of different bacteria	12	22
Number of uncultured bacteria	9 av 68	21 av 61

Figure 27. Overview of bacteria detected.

A total of 28 different bacteria were identified and 5 of these were found in both samples. *Catonella*, *Campylobacter*, *Fusobacterium*, *Peptostreptococcus*, *Streptococcus* and *Treponema* were represented many times. We also recognize from the results that uncultivable species are well represented.

We found this to be a useful method for analyzing bacteria in necrotic bone samples, and seeing that many of the DNA-segments were identical to uncultured bacteria, this method proves especially valuable. We do realize that the high numbers of some bacteria are not necessarily correlated to their representation in the original samples, since a random selection of DNA segments are included in the droplet added to the first PCR- reaction and later a selection of these are inserted into the cloning cells.

To see how our results fits into other findings we made a search in Pub-med on ORN and the different bacteria and their role in systemic disease and some of the interesting findings are mentioned.

- Støre et al. (13) found polymicrobial bacterial infection in deep medullary bone of ORN where rods, spirochetes and cocci were present and rods were the predominant.

- In reviewing 60 patients suffering from ORN (28) and Osteomyelitis (32), Calhoun et al. (18) reported the most commonly found bacteria to be, *Streptococcus* sp., *Bacteroides* sp., *Lactobacillus* sp., *Eubacterium* sp. and *Klebsiella* sp. Only four cultures were positive for *Actinomyces* (13).

- Støre et al. (14) found *Porphyromonas gingivalis* to be the predominant organism in most of their material and also found *Actinomyces* species to be present in all of the samples. In a study involving 31 patients, Hansen et al. (15) suggests *Actinomyces* species play a significant role in development of osteoradionecrosis as they could be found in 20 of their patients. In another report they also found a relationship between the presence of *Actinomyces* spp. and an unfavourable treatment outcome (16). *Actinomyces* has not yet been found in our material.



The different options for bacteria to enter bone tissue would be by superficial contamination, endodontal or periodontal infection or through a Haematological pathway. It is well known that bacteremia may occur and sometimes have devastating effects. We will now look at some points that may suggest bacteria are not only a secondary infection or contamination, but a contributing factor.

- Using DNA-DNA hybridization technique, Støre et al. were able to detect presence of bacteria in 9 samples from medullary bone which had been covered by mucoperiost (14). Intact mucosa supports a different pathway for bacteria than superficial contamination.
- Epidemiological studies show that 14-20% of bacterial endocarditis are of oral origin (17). The most common are *Streptococcus* but *Eikenella corrodens* has also been found.

## **Conclusion**

From the preliminary results we conclude that there is a high bacterial diversity associated with osteoradionecrosis. Bacteria that dominate the bacterial flora are mainly of oral origin. Known periodontal pathogens such as *Treponema* spp. and *Porphyromonas gingivalis* are well represented. Further studies on bacterial flora associated with osteoradionecrosis may contribute to a more precise use of antibiotics.

Result of this study will be submitted for publication in Journal of Clinical Microbiology.

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The pictures of PCR and cloning techniques are taken from [www.sumanasinc.com](http://www.sumanasinc.com)

The pictures of sequencing techniques are taken from [www.Wiley.com](http://www.Wiley.com)